Neurobiology of Disease 44 (2011) 292–303

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

Cleavage of the vesicular glutamate transporters under excitotoxic conditions

Andrea C. Lobo [a,b], João R. Gomes [a,b,1], Tatiana Catarino [a,b,1], Miranda Mele [a,b,1], Pedro Fernandez [a,b,1], Ana R. Inácio [c], Ben A. Bahr [d], Armanda E. Santos [a,e], Tadeusz Wieloch [c], Ana Luísa Carvalho [a,b], Carlos B. Duarte [a,b,*]

[a] Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal
[b] Department of Life Sciences, University of Coimbra, Coimbra, Portugal
[c] Wallenberg Neuroscience Center, Lund University, Lund, Sweden
[d] Biotechnology Research and Training Center, University of North Carolina-Pembroke, Pembroke, NC, USA
[e] Faculty of Pharmacy, University of Coimbra, Coimbra, Portugal

ARTICLE INFO

Article history:
Received 28 March 2011
Revised 21 June 2011
Accepted 11 July 2011
Available online 21 July 2011

Keywords:
Brain ischemia
Calpains
Excitotoxicity
Oxygen and glucose deprivation
Vesicular glutamate transporters

ABSTRACT

Glutamate is loaded into synaptic vesicles by vesicular glutamate transporters (VGLUTs), and alterations in the transporters expression directly regulate neurotransmitter release. We investigated changes in VGLUT1 and VGLUT2 protein levels after ischemic and excitotoxic insults. The results show that VGLUT2 is cleaved by calpains after excitotoxic stimulation of hippocampal neurons with glutamate, whereas VGLUT1 is downregulated to a lower extent. VGLUT2 was also cleaved by calpains after oxygen/glucose deprivation (OGD), and downregulated after middle cerebral artery occlusion (MCAO) and intrahippocampal injection of kainate. In contrast, VGLUT1 was not affected after OGD. Incubation of isolated synaptic vesicles with recombinant calpain also induced VGLUT2 cleavage, with a little effect observed for VGLUT1. N-terminal sequencing analysis showed that calpain cleaves VGLUT2 in the C-terminus, at Asn^{534} and Lys^{542}. The truncated GFP-VGLUT2 forms were found to a great extent in non-synaptic regions along neurites, when compared to GFP-VGLUT2. These findings show that excitotoxic and ischemic insults downregulate VGLUT2, which is likely to affect glutamatergic transmission and cell death, especially in the neonatal period when the transporter is expressed at higher levels.

Introduction

Glutamate plays a critical role in the pathophysiology of cerebral ischemia (Doyle et al., 2008). Excitotoxicity caused by overactivation of glutamate receptors contributes to neuronal death in several neuronal pathologies, including ischemia, epilepsy and neurodegenerative disorders (Szydlowska and Tymianski, 2010). The $[Ca^{2+}]_i$ overload resulting from the overactivation of glutamate receptors induces an excessive stimulation of calpains, a family of cysteine proteases activated by calcium, leading to the cleavage of several substrates (Bevers and Neumar, 2008). These include proteins that play key roles in glutamatergic synapses, including AMPA (Yuen et al., 2007) and NMDA receptor subunits (Gascon et al., 2008), and mGluR1 metabotropic glutamate receptors (Xu et al., 2007). Caspase activation also contributes to neuronal death in brain ischemia (Broughton et al., 2009), and these proteases mediate the degradation of AMPA receptor subunits during early periods of cell stress. The resulting downregulation of excitatory activity may ensure apoptosis by preventing excitotoxic necrosis (Glazner et al., 2000).

The activity of glutamatergic synapses is regulated by plasma membrane and vesicular glutamate transporters. The reversal of plasma membrane-associated glutamate transporters is thought to contribute to excitotoxic neuronal damage in brain ischemia (Chao et al., 2010). Changes in the abundance of vesicular glutamate transporters (VGLUT) were also reported in brain ischemia. VGLUT2 is downregulated in the CA1 layer of the gerbil hippocampus after a mild transient period of global ischemia, and this change was associated with delayed neuronal death (Kim et al., 2006). Similarly, VGLUT2 and VGLUT3 were also found to be downregulated in the cerebral cortex and caudate–putamen of rats subjected to transient MCAO. In contrast, VGLUT1 protein levels were transiently increased in the same brain regions during the first 3 days of reperfusion, but decreased 7 days after the ischemic insult (Sanchez-Mendoza et al., 2010). VGLUT1 and VGLUT2 account for the ability of most excitatory neurons to release glutamate by exocytosis (Fremeau et al., 2004). VGLUT1 levels increase gradually after birth, becoming the dominant form in the adult brain, whereas VGLUT2 shows a higher expression early in development (Boulland et al., 2004). Regulation of VGLUT expression may affect quantal size (Wojcik et al., 2004) and, therefore,

* Corresponding author at: Center for Neuroscience and Cell Biology, Department of Life Sciences, University of Coimbra, 3004-517 Coimbra, Portugal. Fax: +351 239 822776.
E-mail address: cbduarte@ci.uc.pt (C.B. Duarte).
[1] These authors contributed equally to this work.
Available online on ScienceDirect (www.sciencedirect.com).

0969-9961/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.nbd.2011.07.010

changes in their expression may affect glutamate release in brain ischemia, particularly in the penumbra region where ATP is available to allow exocytosis to occur.

In this work we further investigated the effects of excitotoxic stimulation and *in vivo* and *in vitro* brain ischemia in the vesicular glutamate transporter protein levels. The results show that VGLUT2 is particularly sensitive to excitotoxic stimulation, and the cleavage of the transporter by calpains gives rise to a truncated form that is not targeted to synapses.

Material and methods

DNA constructs

To clone the VGLUT2 C-terminal in pGEX6P2, the cDNA encoding the C-terminal of rat (*Rattus norvegicus*) VGLUT2 was obtained by RT-PCR from rat cerebral cortex RNA. The first strand cDNA was produced using the 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche), and the resulting cDNA was amplified by PCR using primers forward 5′-CGGAATTCTATTTGCCTCAGGAGAGAAG-3′ and reverse 5′-CCGCTCGAGT-TATGAATAATCATCTCGGT-3′. This product was cloned into pGEM vector (Promega), and then subcloned into pGEX6P2 using EcoRI and XhoI restriction sites, in frame with glutathione S-transferase (GST). The pGEX-VGLUT2 C-terminus sequence was confirmed by DNA sequencing reactions. The plasmids encoding for the fusion proteins of GST-VGLUT2 C-terminal mutants, D522A and D525A, were produced by site-directed mutagenesis, using PCR.

The rat VGLUT2 in pCMV6b was a kind gift from J. Takeda (Gifu University, Japan). The plasmid encoding VGLUT2 rat protein fused with green fluorescent protein (GFP) was generated by molecular cloning. The cDNA of rat VGLUT2 was amplified by PCR using the primers forward 5′-CGGAATTCTATGGAGTCGGTAAAACAAAG-3′ and reverse 5′-CGGGATCCTTATGAATAATCATCTCGG-3′, and subcloned into pGEM vector (Promega) through EcoRI and BamHI restriction sites. VGLUT2 was then subcloned into pEGFPC1 (BD Biosciences Clontech) in frame with GFP. The pEGFPC1-VGLUT2 sequence was verified by DNA sequencing reactions. The truncated pGFPC1-VGLUT2 plasmids were generated by insertion of stop codons after amino acids 533 or 541 by directed mutagenesis, using PCR.

The rat VGLUT1 in pcDNA3.1(+) at HindIII/XhoI site was a kind gift of S. Takamori (Tokyo University, Japan). The plasmid encoding the GST-VGLUT1 C-terminus fusion protein was produced by PCR using the primers forward 5′-CCGGAATTCTCGGGAGAGAAACAGCCGTGG-3′ and reverse 5′-CCGCTCGAGTCAGTAGTCCCGGACAGGGGG-3′ to amplify VGLUT1 C-terminus. The PCR product was cloned into pGEX4T1 vector (Pharmacia) with EcoRI and XhoI. The pGEX4T1-VGLUT1 C-terminus sequence was confirmed by DNA sequencing reactions.

Hippocampal cultures

High density cultures of rat hippocampal neurons were prepared from E18–E19 Wistar rat embryos as previously described (Gomes et al., 2011). Neuronal cultures were maintained in serum-free Neurobasal medium (Gibco Invitrogen), supplemented with B27 (Gibco Invitrogen), glutamate (25 μM), glutamine (0.5 mM) and gentamycin (0.12 mg/ml). Cells were cultured at a density of 9×10^4 cells/cm^2 on poly-D-lysine coated 6-well microplates (MW6), or at 2×10^5 cells/cm^2 on poly-D-lysine coated coverslips (10 mm), and kept at 37 °C in a humidified incubator with 5% CO_2/95% air, for 7 or 15 days *in vitro* (DIV).

Low density hippocampal cultures were prepared as previously described (Goslin et al., 1998). Briefly, hippocampi were dissected from E18 rat embryos and dissociated using trypsin (0.25%). Neurons were plated at a final density of $1–5\times10^4$ cells/dish (60 mm culture dishes) on poly-D-lysine-coated coverslips and cultured in the presence of an astroglial feeder layer. Cultures were maintained in Neurobasal medium supplemented with B27 supplement (1:50), 25 μM glutamate, 0.5 mM glutamine and 0.12 mg/ml gentamycin. To prevent the overgrowth of the glia, neuron cultures were treated with 5 μM cytosine arabinoside after 3 DIV and maintained in a humidified incubator with 5% CO_2/95% air, at 37 °C, for up to 2 weeks, feeding the cells once per week by replacing one-third of the medium.

Excitotoxic stimulation with glutamate

Hippocampal neurons (7 DIV) were exposed to 125 μM glutamate for 20 min in Neurobasal medium and further incubated in culture conditioned medium for the indicated periods of time. Pre-incubations of 2 h were used when cells were treated with the calpain inhibitors MDL28170 (Calbiochem) and ALLN (Calbiochem; 50 μM), or with the pan-caspase inhibitor Z-VAD-FMK (Biomol; 50 μM). Under control conditions, neurons were not exposed to glutamate.

Induction of apoptosis

Hippocampal neurons (7 DIV) were exposed to 30 nM staurosporine for 24 h (Prehn et al., 1997), or to 300 nM for 10 min (Lankiewicz et al., 2000), and further incubated in culture conditioned medium for 24 h. Withdrawal of trophic factor support was accomplished by replacing culture medium with Locke's buffer for 48 h, as previously described (Guo et al., 2008).

Oxygen–glucose deprivation (OGD)

Hippocampal neurons (15 DIV) were incubated in a glucose-free saline buffer (116 mM NaCl, 25 mM sucrose, 10 mM Hepes, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4, 1.8 mM $CaCl_2$, 25 mM $NaHCO_3$) under an anaerobic atmosphere (10% H_2, 85% N_2, 5% CO_2) (Forma Anaerobic System, Thermo Fisher Scientific), at 37 °C for 1.5 h. After the OGD challenge, the buffer was replaced by culture conditioned medium and the cells were returned to the humidified 5% CO_2/95% air incubator for 8 h. Control neurons (Sham) were incubated in the saline buffer described above, supplemented with 25 mM glucose (in the absence of sucrose), and kept in the humidified 5% CO_2/95% air incubator at 37 °C. Pre-incubations of 2 h were used when cells were treated with the calpain inhibitor ALLN (50 μM), and the inhibitor was also present during the post-incubation period. For nuclear morphology analysis hippocampal neurons (15 DIV) were subjected to OGD for 1.5 h followed by 12 h of post-incubation in culture conditioned medium. After fixation the cells were incubated with 1 μg/ml Hoechst 33342 for 10 min. Three coverslips were prepared for each experimental condition, and at least 200 cells were counted in each case.

Preparation of extracts

Hippocampal culture extracts were prepared as previously described (Gomes et al., 2011). Briefly, neurons were washed with ice-cold PBS, and lysed with RIPA. After centrifugation, protein in the supernatants was quantified using the Bicinchoninic acid (BCA) assay kit (Pierce), and the samples were denaturated with 2× concentrated denaturating buffer (125 mM Tris, pH 6.8, 100 mM glycine, 4% SDS, 200 mM DTT, 40% glycerol, 3 mM Na_3VO_4, and 0.01% bromophenol blue). Extracts used for VGLUT1 and VGLUT2 analysis were not boiled to avoid VGLUT aggregation.

Western blot

Protein samples were separated by SDS-PAGE, in 12% polyacrylamide gels, transferred to polyvinylidene fluoride (PVDF) membranes (Millipore), and immunoblotted as previously described (Gomes et al., 2011). Blots were incubated with primary antibodies overnight at 4 °C, washed, and exposed to alkaline phosphatase-conjugated secondary antibodies (1:20,000 dilution for anti-rabbit IgG and 1:10,000 dilution

for mouse IgG) for 1 h at room temperature. Alkaline phosphatase activity was visualized by enhanced chemifluorescence (ECF) on the Storm 860 Gel and Blot imaging system, and quantified using the ImageQuant software (GE Healthcare). The following primary antibodies were used: anti-VGLUT1 (1:5000, Synaptic Systems), anti-VGLUT2 (1:1000, Ab:510-582aa; Synaptic Systems), anti-VGLUT2 (1:750, Ab:N-terminal; USBiological), anti-GST (1:2000, GE Healthcare), anti-α-spectrin (1:1000, Millipore), anti-active caspase-3 (1:1000, Cell Signaling Technology) and anti-GFP (1:2000, Abcam). The anti-synaptophysin (1:10,000, Synaptic Systems or Sigma) antibody was used as loading control.

Neuron transfection with calcium phosphate

Constructs were recombinantly expressed in primary cultures of hippocampal neurons using a calcium phosphate co-precipitation method as previously described (Gomes et al., 2011). After transfection the cells were further incubated in culture conditioned medium for 48–72 h to allow protein expression.

Immunocytochemistry

Hippocampal neurons were fixed in 4% sucrose/paraformaldehyde (in PBS) and permeabilized with 0.3% Triton X-100 in PBS. The neurons were incubated with 10% BSA in PBS for 30 min at 37 °C, and incubated with primary antibodies diluted in 3% BSA in PBS, overnight at 4 °C. The cells were washed with PBS, and incubated with the appropriate secondary antibodies, for 1 h at 37 °C. The coverslips were mounted in a fluorescence mounting medium (DAKO). Imaging was performed on a Zeiss Axiovert 200 M fluorescence microscope, using a 63×1.4NA oil objective. The primary antibodies used were anti-VGLUT2 (1:200 Ab: 510–582; Synaptic Systems), anti-GFP (1:200, MBL [anti-rabbit] or Roche [anti-mouse]), anti-Synapsin (1:200, Millipore) and anti-PSD95 (1:200, Pierce). The secondary antibodies used were Alexa Fluor 488, 594 and 647 (Invitrogen). Controls lacking specific antibodies confirmed no detectable immunolabeling with the secondary antibodies. Images were quantified using the image analysis software ImageJ. For each set of experiments the analyzed cells were cultured, stained and imaged using identical settings. Images were acquired as gray scale from individual channels and pseudocolor overlays were prepared using ImageJ software. To quantify the VGLUT2 signal, fields for imaging were chosen by the GFP channel to check for the presence of transfected neurons. To quantify GFP-VGLUT2 clusters, the digital images were subjected to a user-defined intensity threshold to select clusters and measured for cluster intensity, number, and area for the regions of interest. The number of synaptic clusters was determined as the number of clusters overlapping thresholded and dilated synapsin or PSD95. Regions around thresholded GFP-VGLUT2 puncta were overlaid as a mask in the synapsin or in the PSD95 channel, and colocalization was determined. Measurements were performed in three independent preparations for analysis of colocalization with synapsin, and three other independent preparations for analysis of colocalization with PSD95, and at least 18 cells per experimental condition were analyzed for each preparation.

Intra-hippocampal injection of kainate

Intra-hippocampal injection of kainate was performed as previously described (Takano et al., 2005), with minor modifications. Briefly, wild type adult male mice (C56BL6) were anesthetized with avertin (2,2,2 tribromoethanol, 2-methyl-2-butanol), placed in a stereotaxic apparatus, and given a unilateral injection of 1 nmol of kainate (in 0.3 μl of PBS) into the hippocampal CA1 region, using a 10 μl motorized syringe (Hamilton). The coordinates of the injection were anterior–posterior: −2.3 mm, medial–lateral: −1.5 mm, and dorsal–ventral: −1.7 mm from the bregma. Kainate was injected at a constant flow rate of 0.05 μl/min. The death rate in this experiment was less than 5%. Four, 8 or 12 h after injection mice were sacrificed and a 2 mm section around the hippocampus was taken using a 1 mm coronal mouse matrix. The slices were frozen with dry ice and the contralateral and the damaged ipsilateral areas of the hippocampal slices were taken using a Harris Unicore 2 mm tip (Pelco International). Samples were then homogenized and processed for western blot.

Middle cerebral artery occlusion (MCAO)

Focal cerebral ischemia was induced by transient occlusion of the right middle cerebral artery for 45 min, using the intraluminal filament placement technique as described previously (Nygren and Wieloch, 2005). Mice were sacrificed 24 h after the occlusion and 1 mm sections were done using a 1 mm coronal mouse matrix. The slices were frozen with dry ice and the damaged ipsilateral and contralateral areas of the slices were taken using a Harris Unicore 2 mm tip (Pelco International). Samples were then homogenized and processed for western blot. These experiments were conducted according to protocols approved by the Mälmo/Lund Ethical Committee for Animal Research.

Expression of recombinant proteins

To produce the GST-VGLUT2 C-terminus and the GST-VGLUT1 C-terminus fusion proteins, the plasmids were transformed into *E. coli* BL21 and protein expression was induced by treating the *E. coli* culture in the exponential phase of growth ($A_{600nm} = 0.8–2$) with 0.5 mM isopropyl β-D-thiogalactoside (IPTG), for 30 min at 30 °C. The protein was extracted and purified from the bacterial pellet through affinity chromatography on glutathione Sepharose 4B (GE Healthcare), according to the manufacturer's recommendations. The purified proteins were analyzed in SDS-PAGE gels and stained with Coomassie Blue.

In vitro assays with recombinant calpain

Incubation of GST fusion proteins with calpain

GST-VGLUT1 C-terminus and GST-VGLUT2 C-terminus fusion proteins (100 μg) were diluted in 50 mM Tris–HCl (pH 7.4), 100 mM KCl, 2 mM DTT and 2.5 mM of $CaCl_2$ (or 1 mM EDTA), and incubated with 2.5 U/ml of recombinant calpain I (human erythrocytes, Calbiochem), for 15 min at 37 °C. Incubation was stopped using concentrated denaturating buffer and samples were boiled for 5 min at 95 °C. The peptides were separated by SDS-PAGE Tricine gels. The gels were stained with Coomassie Blue colloidal solution (Candiano et al., 2004). The same experimental procedure was done to transfer the proteins to a PVDF membrane, and the bands indicated were analyzed by N-terminal Edman sequencing, at the Protein Sequencing facility of the University of Leeds, UK.

Incubation of synaptic vesicles with calpain

Cerebrocortical synaptic vesicles were isolated from the rat brain as previously described (Synaptic Systems—protocol for preparation of P2 and LP2 fractions). The vesicles (5 μg) were diluted in 50 mM Tris–HCl (pH 7.4), 100 mM KCl, 2 mM DTT and 2.5 mM of $CaCl_2$ (or 1 mM EDTA), and incubated with 2.5 U/ml of recombinant calpain I, for the indicated periods of time at 37 °C. Incubation was stopped with concentrated denaturation buffer. When appropriate, calpain inhibitors MDL28170 (50 μM) or ALLN (50 μM) were added during the assay.

In vitro caspase-3 assay

GST-VGLUT2 C-terminus fusion protein (5 μg) was diluted in 20 mM PIPES, 100 mM KCl, 1 mM EDTA, 10% sucrose and 10 mM DTT (with or without 0.1% CHAPS), and incubated with 2 U/ml of recombinant caspase-3 for 20 h at 37 °C. Incubation was stopped using concentrated denaturation buffer and samples were boiled for 5 min at 95 °C. When appropriate, the caspase-3 inhibitor Z-DEVD-FMK (30 μM, Biomol) was added during the assay.

Statistical analysis

The immunoreactivity obtained in each experimental condition was calculated as a percentage of the control. Data are presented as mean ± SEM of at least three different experiments, performed in independent preparations. Statistical analysis of the results was performed using one-way ANOVA analysis followed by either Dunnett's or Bonferroni's post test, or unpaired Student's *t*-test: n.s. non significant, ***$p<0.001$, **$p<0.01$, *$p<0.05$.

Results

VGLUT1 and VGLUT2 are cleaved after glutamate excitotoxic stimulation

To assess the effect of excitotoxic stimulation on VGLUT1 (~60 kDa) and VGLUT2 (~60 kDa) protein levels, cultured hippocampal neurons (7 DIV) were stimulated with 125 μM of glutamate for 20 min, and further incubated in culture conditioned medium for different periods of time. VGLUT protein levels were analyzed by western blot using antibodies against the C-terminal region of both transporters. Stimulation of cultured hippocampal neurons under these conditions causes 40–50% of cell death (Almeida et al., 2005). The results show a time-dependent downregulation of both transporters, although the effects on VGLUT2 were observed at an earlier time point after the glutamate stimulation and were more robust than those observed for VGLUT1 (Figs. 1A and B). In fact, VGLUT2 protein levels decreased by about 70% 12 h after the insult, whereas VGLUT1 protein levels dropped by 35%. In contrast, no effect on the synaptophysin protein levels was observed, showing that not all vesicular proteins are deregulated following excitotoxic stimulation.

VGLUT2 cleavage induced by glutamate was also investigated using an antibody against its N-terminus (Fig. 1C). This analysis showed that downregulation of VGLUT2 protein levels is associated with the time-dependent formation of a stable cleavage product (tVGLUT2) of approximately 55 kDa. This cleavage product was also observed in cultured hippocampal neurons transfected with a GFP-VGLUT2 fusion

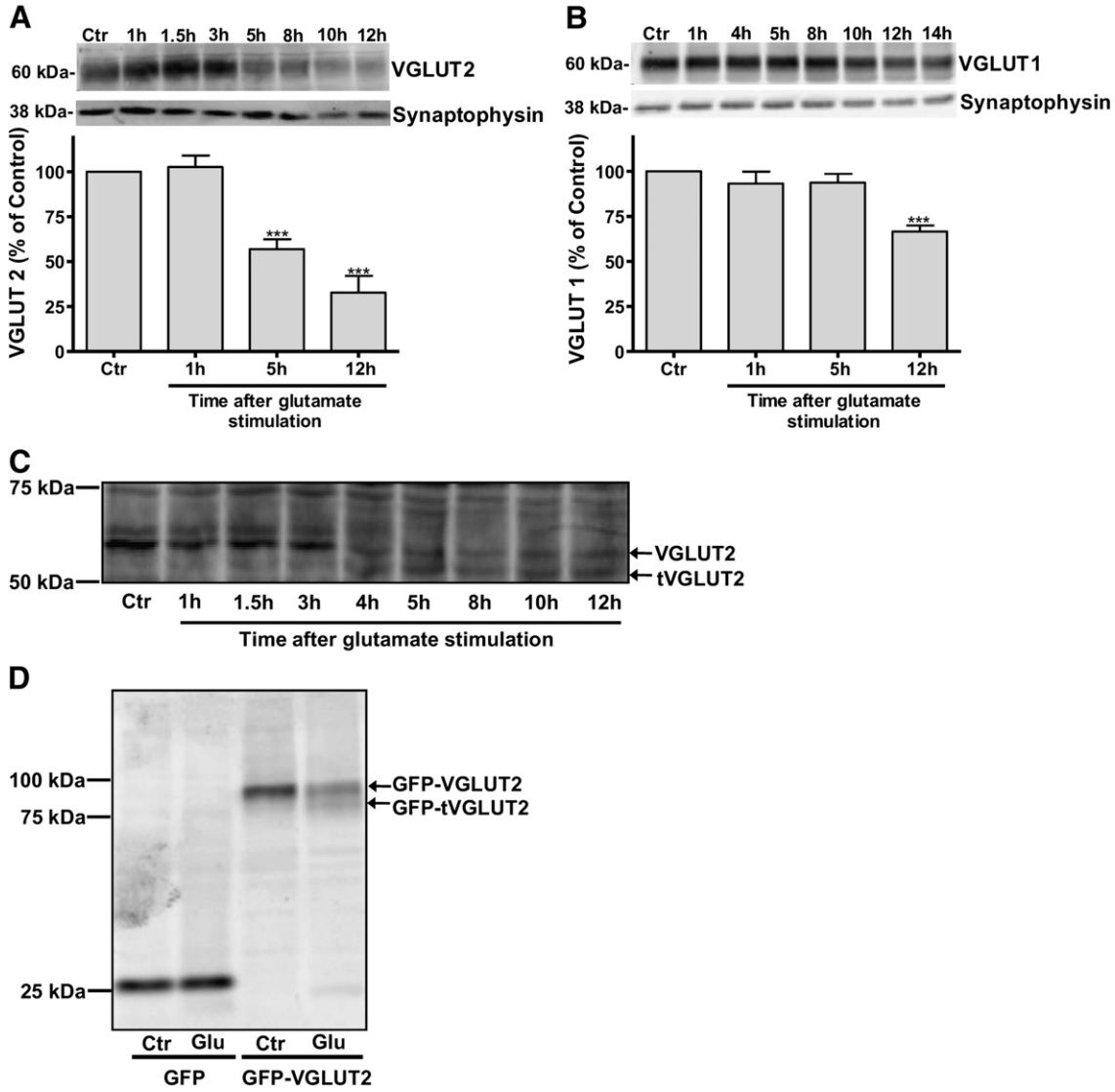

Fig. 1. Glutamate-induced cleavage of VGLUT1 and VGLUT2 in cultured hippocampal neurons. Neurons were subjected to excitotoxic stimulation with glutamate (125 μM; 20 min) and the extracts were prepared after incubation in culture conditioned medium for the indicated periods of time. Samples were analyzed by western blot with antibodies against the C-terminal region of VGLUT2 (A) or VGLUT1 (B), and against the N-terminus of VGLUT2 (C). The immunoreactivity with an antibody against synaptophysin was used as a loading control. Control (Ctr) VGLUT protein levels determined in cells not exposed to glutamate were set to 100%. The results are the average ± SEM of 4 to 5 independent experiments performed in distinct preparations. Statistical analysis was performed using One Way ANOVA, followed by the Dunnett's multiple comparison test performed for each condition as compared to the control condition (***$p<0.001$). (D) Cultured hippocampal neurons were transfected with GFP-VGLUT2 and stimulated with glutamate (125 μM; 20 min), followed by 8 h of incubation in culture conditioned medium. Cell extracts were analyzed by western blot using an antibody against GFP. The results represent two independent experiments performed in different preparations.

protein and subjected to excitotoxic stimulation with glutamate; in this case western blot was performed with an anti-GFP antibody. GFP is not cleaved after the toxic insult with glutamate (Fig. 1D).

VGLUT2 is selectively downregulated under excitotoxic conditions in vitro and in vivo

Transient incubation of cultured neurons under oxygen and glucose deprivation (OGD) is an *in vitro* model of transient global ischemia (Hertz, 2008). Cultured hippocampal neurons were subjected to OGD for 1.5 h, which induces 30.0% ± 3.7 (n = 4) of cell death as determined 12 h after the stimulus using the Hoechst 33342 staining. VGLUT1 and VGLUT2 protein levels were analyzed 8 h after OGD. The results show a selective downregulation of VGLUT2 (70% decrease) after OGD, whereas VGLUT1 or synaptophysin protein levels were not affected (Fig. 2A).

To determine if VGLUT1 and VGLUT2 cleavage also occurs under excitotoxic conditions *in vivo*, adult mice were subjected to intra-hippocampal injection of kainate and the protein levels of both transporters were determined by western blot at different time points after the insult. Kainate injection decreased VGLUT2 protein levels in the ipsilateral hemisphere (as compared to the contralateral hemisphere) by about 20% when determined 12 h after the insult, but no differences were observed for the other time points tested. Surprisingly, an increase of about 40% in VGLUT1 protein levels was observed in the ipsilateral hemisphere 8 h after kainate injection, compared to the contralateral hemisphere levels, with no differences observed at 4 or 12 h after the injection (Fig. 2B). Furthermore, the excitotoxic insult

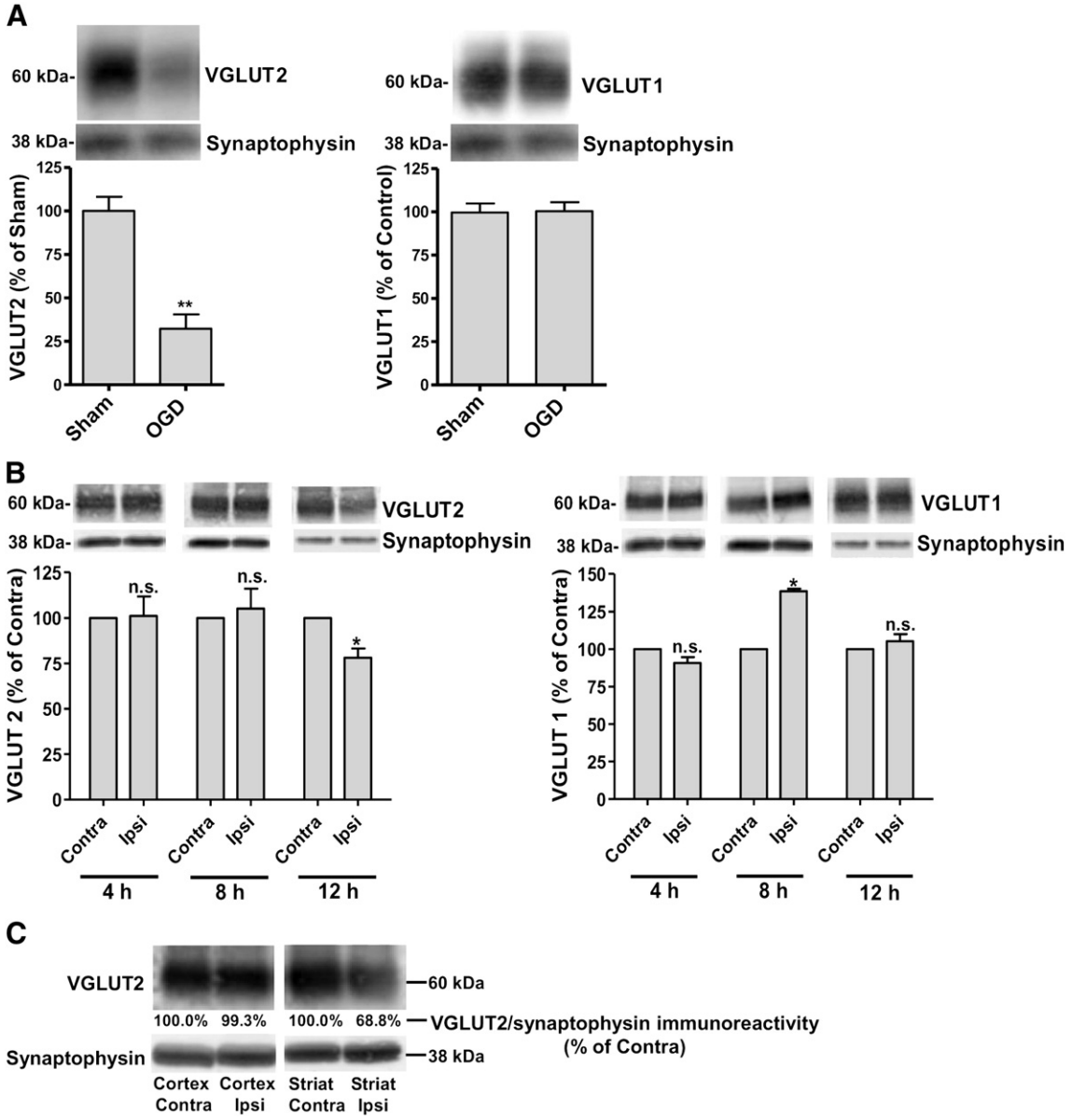

Fig. 2. VGLUT2 is cleaved under excitotoxic conditions *in vitro* and *in vivo*. (A) Cultured hippocampal neurons were subjected to OGD for 1.5 h and further incubated in culture conditioned medium for 8 h. VGLUT1, VGLUT2 and synaptophysin protein levels were assessed by western blot. The results are the average ± SEM of 3 independent experiments performed in different preparations. (B) Adult mice were injected with 1 nmol kainate in the right hippocampus and 4, 8 or 12 h later the ipsilateral and the contralateral hippocampi were collected. Hippocampal extracts were analyzed by western blot using antibodies against VGLUT1, VGLUT2 and synaptophysin, and the results are the average ± SEM of 3 to 5 independent experiments, performed in different animals. Statistical analysis was performed using Unpaired Student's *t*-test (A) or using One Way ANOVA followed by Bonferroni's multiple comparison test (B). n.s.—not significant, $^{*}p<0.05$, $^{**}p<0.01$ as compared with the Sham condition (A) or with the contralateral hemisphere (B). (C) Adult mice were subjected to a transient 45 min MCAO. VGLUT2 and synaptophysin levels were determined in the cerebral cortex and striatum (Striat) of the ipsilateral (Ipsi) and contralateral (Contra) brain hemispheres, 24 h after the lesion, by western blot. The ratio between VGLUT2 levels and the loading control (synaptophysin) was determined. The ratio in the contralateral hemisphere was set to 100%. The results represent two independent experiments performed in different animals.

did not affect synaptophysin protein levels. Control experiments, consisting in the injection of PBS, the kainate vehicle, did not change VGLUT1 or VGLUT2 protein levels (data not shown).

A decrease in VGLUT2 protein levels was also observed in the striatum of adult mice subjected to transient middle cerebral artery occlusion (MCAO). Brain extracts were prepared from the ipsilateral and contralateral brain hemispheres (cerebral cortex and striatum) 24 h after a 45 min period of MCAO. A decrease on VGLUT2 protein levels was observed in the striatum, but not in the cerebral cortex, as assessed by western blot using the antibody against the VGLUT2 C-terminus. As expected, this change in VGLUT2 protein levels was observed in the ipsilateral but not in the contralateral hemisphere (Fig. 2C). Synaptophysin protein levels were used as loading control.

Calpain cleaves VGLUT2 under in vitro excitotoxic conditions

To investigate whether calpains are involved in VGLUT2 cleavage after excitotoxic stimulation with glutamate, cultured hippocampal neurons were pre-incubated with the calpain chemical inhibitors ALLN or MDL28170, and subjected to the glutamate insult (125 μM; 20 min) in the presence of the inhibitors. VGLUT2 protein levels were analyzed by western blot using the antibody raised against the C-terminus of the transporter, 5 h after glutamate stimulation. Both calpain inhibitors prevented the decrease on VGLUT2 levels induced by glutamate (Fig. 3A). ALLN also abrogated VGLUT2 downregulation 8 h after the OGD (1.5 h) insult (Fig. 3B). These results indicate that VGLUT2 is cleaved by calpains after excitotoxic or ischemic *in vitro* insults.

In order to confirm VGLUT2 cleavage by calpains, isolated cerebrocortical synaptic vesicles were incubated with recombinant calpain I in a Ca^{2+}-containing medium, and VGLUT2 protein levels were determined by western blot at different incubation periods (5, 10, 15 and 60 min). These experiments showed a downregulation of VGLUT2 by calpain I, and the effect was inhibited by MDL28170 and ALLN (Fig. 3C). Also, no decrease in VGLUT2 protein levels was observed in experiments where synaptic vesicles were incubated with calpain in a Ca^{2+}-free medium to prevent the activity of the protease. Taken together, these evidences show that VGLUT2 is a calpain substrate. Although a decrease in VGLUT1 protein levels was also observed (Fig. 3D), the effect was small when compared to that observed for VGLUT2. Thus, incubation of synaptic vesicles with calpain for 10 min decreased VGLUT2 protein levels by

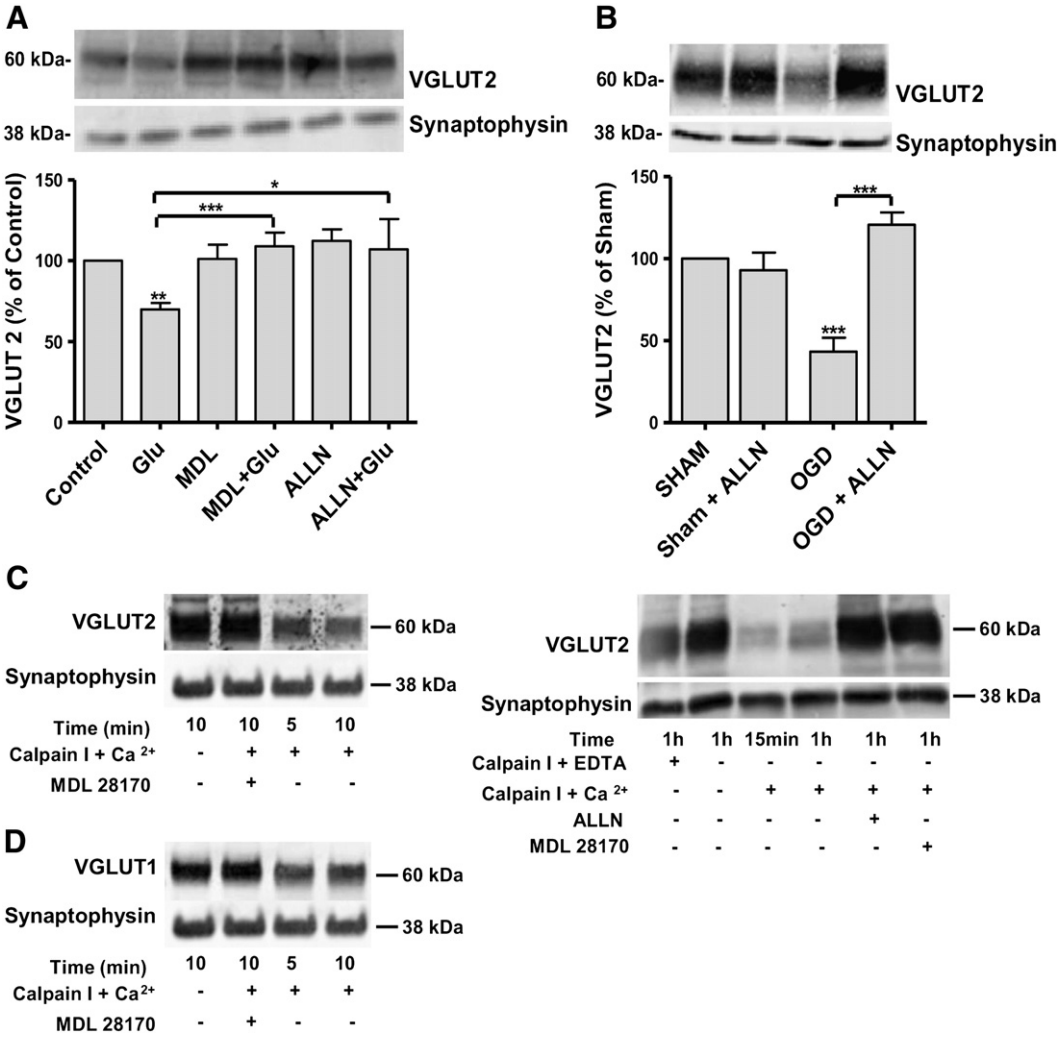

Fig. 3. VGLUT2 is cleaved by calpains. Cultured hippocampal neurons were stimulated with glutamate (125 μM; 20 min), and further incubated in culture conditioned medium for 5 h (A), or subjected to OGD for 1.5 h and further incubated in culture conditioned medium for 8 h (B). Where indicated the cells were pre-incubated with MDL28170 or ALLN and the inhibitors were also present throughout the experiment. VGLUT2 and synaptophysin protein levels were determined by western blot. Control (A) or sham (B) VGLUT2 protein levels were set to 100%. The results are the average ± SEM of 3 to 9 independent experiments performed in distinct preparations. Statistical analysis was performed using One Way ANOVA, followed by the Bonferroni's multiple comparison test (*$p<0.05$,**$p<0.01$,***$p<0.001$). (C and D) Cerebrocortical synaptic vesicles (5 μg) were incubated with recombinant calpain I (2.5 U/ml) for the indicated periods of time at 37 °C, in the presence (2.5 mM Ca^{2+}) or in the absence (1 mM EDTA) of calcium. Where indicated the effect of the calpain inhibitors MDL 28170 (50 μM) or ALLN (50 μM) was tested. Incubation of synaptic vesicles with calpain for 5 min, 10 min or 60 min decreased VGLUT2 protein levels by 50%, 66% and 73%, respectively. Extracts were analyzed by western blot using antibodies against VGLUT2, VGLUT1 or synaptophysin.

66%, whereas VGLUT1 was reduced by about 30%. As expected, no effects were observed on the synaptophysin protein levels.

VGLUT2 is cleaved by calpain in two different sites in the C-terminal region

VGLUT2 is thought to possess 12 transmembrane domains (transmembrane helices as determined with the protein prediction online software—TMHMM—by the Center of Biological Sequence Analysis, Denmark), and the N- and C-termini of the protein are directed towards the cytoplasm (Jung et al., 2006). The presence of a putative PEST sequence (epestfind at http://emboss.sourceforge.net/, amino acids 502 to 515, score: +15.77) in the C-terminus of VGLUT2 (Fig. 4A) suggests that this region might be targeted by calpains, which may cleave neighbor sequences (Rechsteiner and Rogers, 1996; Tompa et al., 2004). To further investigate the putative cleavage site(s) in the C-terminus of VGLUT2, we used the algorithm of Tompa et al. (2004), which allows predicting the amino acid sequences targeted by calpains. The results of Fig. 4B show that the highest scores are found close to the putative PEST sequence, and between amino acids 528 and 553. Since it was proposed that calpains cleave their substrates in rather disordered segments of the proteins, we used the metaPrDOS bioinformatic tool to predict the disorder tendency along VGLUT2 sequence (Ishida and Kinoshita, 2008). The results show that VGLUT2 is more disordered at the N- and C-terminal regions (particularly amino acids 506 to 582) (Fig. 4C).

Since the truncated VGLUT2 was only recognized by the antibody that binds to VGLUT2 N-terminus (Figs. 1A and C), it is probable that calpain cleavage occurs at the VGLUT2 C-terminal region, particularly between amino acids 510 and 582, the epitope of the C-terminus antibody. The probable location of VGLUT2 cleavage site(s) at VGLUT2 C-terminus is further supported by the results showing a Ca^{2+}-dependent cleavage by calpain I of the GST fusion protein with the C-terminus of VGLUT2, as assessed by western blot using an anti-GST antibody (Fig. 4D). Cleavage of the fusion protein gives rise to a truncated product with an apparent molecular weight of 32 kDa (oval shape). To determine the calpain cleavage sites at the C-terminus of VGLUT2, the fusion protein was incubated with recombinant calpain I, the generated peptides were separated by SDS-PAGE and the gel was stained with Coomassie Blue, or transferred to a PVDF membrane before

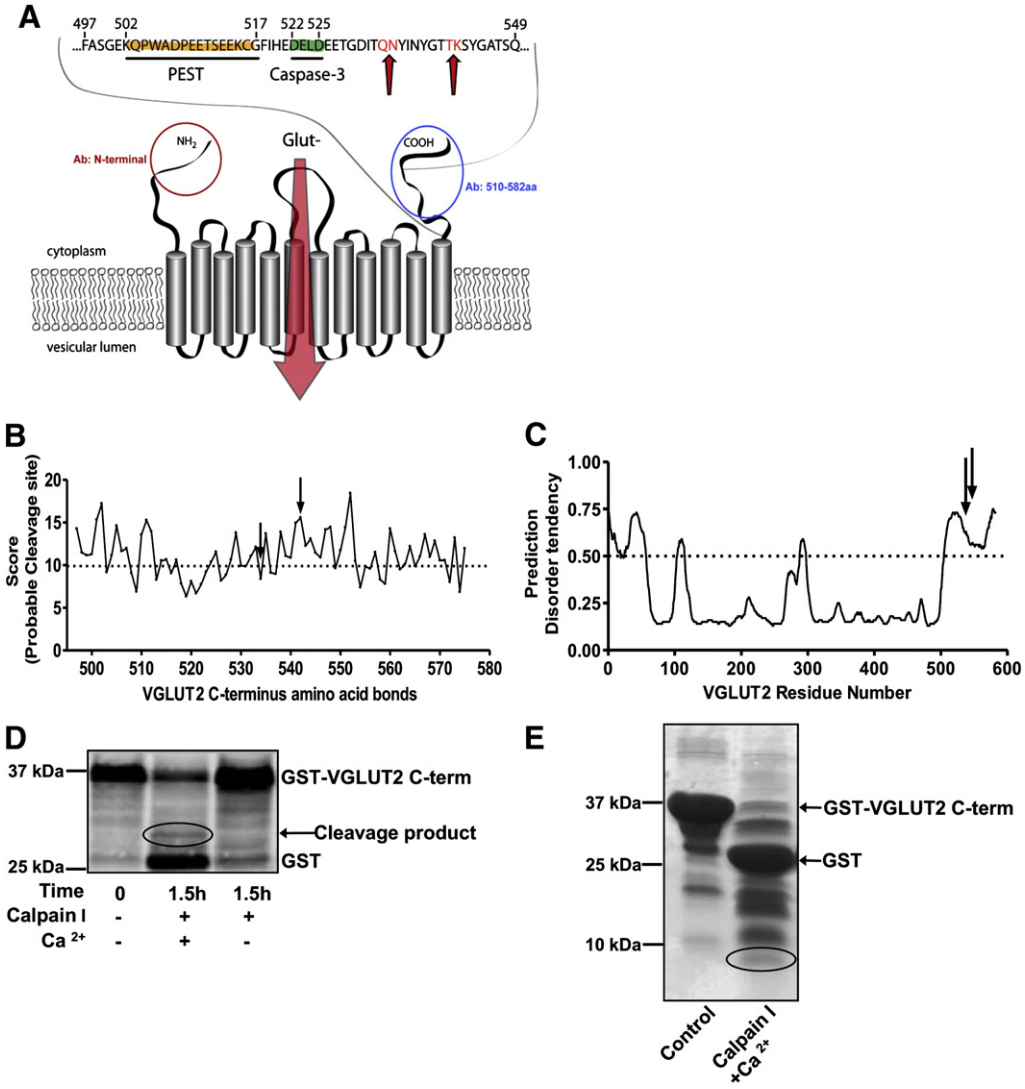

Fig. 4. Identification of the putative calpain cleavage sites of VGLUT2. (A) Predicted topology of VGLUT2 highlighting the epitopes targeted by the antibodies used, the calpain cleavage sites identified (Asn534 and Lys542) and the PEST sequence. (B) Analysis of the probability of cleavage by calpain in the VGLUT2 C-terminus based on the model for prediction of calpain cleavage sites published in Tompa et al. (2004). (C) Prediction of the disorder tendency in VGLUT2 sequence based on a bioinformatic analysis of the amino acid sequence of the protein (Ishida and Kinoshita, 2008). Arrows point to the predicted calpain cleavage sites identified in the present work. (D and E) GST-VGLUT2 C-terminus fusion protein was incubated with recombinant calpain I (2.5 U/ml) for 15 min at 37 °C in the presence or absence of Ca^{2+} (2.5 mM). The peptides were separated by SDS-PAGE and analyzed by western blot using an antibody against GST (D), stained in the gel with Coomassie blue (E), or transferred to a PVDF membrane, stained with Coomassie blue, and processed for N-terminal Edman sequencing. Peptides starting at amino acids Asn534 and Lys542 were identified for the indicated band (E, oval shape). GST is not cleaved by calpain I (data not shown). The results represent four independent experiments performed in different preparations.

staining. The indicated band (oval shape in Fig. 4E) was processed to be sequenced by N-terminal Edman sequencing and two peptides, starting at amino acids Asn534 and Lys542, were identified. The existence of other bands resulting from VGLUT2 cleavage by calpain I (Fig. 4E), which we were not able to identify, raises the possibility that other cleavage sites may exist in the C-terminal region of VGLUT2, upstream of the cleavage sites identified in this work, and may generate shorter forms of VGLUT2.

The predicted topology of VGLUT1 indicates that the protein has a topology similar to VGLUT2, which, together with the presence of two putative PEST sequences (amino acids 494 to 507, score: +10.35, and amino acids 513 to 545, score: +13.25) in its C-terminus, suggests that the transporter could be targeted for calpain cleavage (Supplementary Fig. 1A). The antibody against VGLUT1 binds to an epitope within the protein C-terminus, and only recognizes the full length form of the transporter, suggesting that the cleavage under excitotoxic conditions may occur between amino acids 456 and 560. To evaluate putative cleavage site(s) at VGLUT1 C-terminal, a fusion protein of GST with VGLUT1 C-terminus was incubated with recombinant calpain I and stained with Coomassie Blue. The results show no cleavage of the fusion protein by calpain I, indicating that VGLUT1 is not cleaved by this protease in its C-terminal region (Supplementary Fig. 1D).

Characterization of VGLUT2 cleavage by caspase-3

The VGLUT2 C-terminus also contains a consensus DELD sequence (amino acids 522 to 525) (Fig. 4A), typically targeted by caspase-3 (Chang and Yang, 2000). To investigate if VGLUT2 is cleaved by caspase-3, we performed *in vitro* experiments using the GST-VGLUT2 C-terminus fusion protein incubated with recombinant caspase-3 for 20 h, and the amount of fusion protein was determined by western blot, with an antibody against GST. The results show that VGLUT2 cleavage by caspase-3 gives rise to a product with approximately 32 kDa, which is not produced in the presence of the caspase inhibitor Z-DEVD-FMK (Figs. 5A and B; oval shape). Mutation of the two aspartate residues at positions 522 and 525 to alanine residues prevented GST-VGLUT2 C-terminus protein cleavage, showing that the DELD consensus sequence is targeted by the protease (Fig. 5B). Although it was reported that the presence of CHAPS is important to assay caspase-3 activity *in vitro* (Stennicke and Salvesen, 1997), the detergent was without effect on the cleavage of the GST-VGLUT2 C-terminus fusion protein by caspase-3.

The role of caspases in VGLUT2 downregulation under excitotoxic conditions was addressed by incubating hippocampal neurons with the pan-caspase inhibitor Z-VAD-FMK before subjecting them to excitotoxic stimulation with glutamate. The results show a slight (about 15%) but not significant effect on the cleavage of VGLUT2, 5 h after the insult, indicating that caspases do not play a relevant role in glutamate-evoked VGLUT2 downregulation (Fig. 5C).

The putative effect of caspase-3 on the cleavage of VGLUT2 was further investigated using two stimuli that typically induce apoptotic cell death through activation of this caspase. Hippocampal neurons were exposed to 30 nM staurosporine (STS) for 24 h, a concentration that induces 50% of cell death (Prehn et al., 1997), or to 300 nM STS for 10 min (Lankiewicz et al., 2000). Although STS activated caspase-3, as determined by western blot using an antibody against the cleaved (at Asp185; active) form of the enzyme, no downregulation of VGLUT2 protein levels was observed (Fig. 5D). When deprived from trophic support, cultured hippocampal neurons undergo apoptosis, resulting in the death of around 40% of the neurons during a 48 h period (Guo et al., 2008). Our data show that trophic factor deprivation does not lead to VGLUT2 downregulation, despite the activation of caspase-3 (Fig. 5E). Although we observed VGLUT2 cleavage by caspase-3 *in vitro*, dependent on the DELD sequence, we did not observe VGLUT2 downregulation triggered by cell death-inducing stimuli that result in caspase-3 activation.

VGLUT2 cleavage changes the distribution of the protein along the axons

Since the N- and C-terminal regions of VGLUT2 face the cytoplasm, both regions may play a role in the trafficking of the transporter through interaction with cytoplasmic proteins. In order to understand how VGLUT2 cleavage affects the subcellular localization of the protein, we compared the distribution of the GFP fusion protein with full-length VGLUT2 or truncated forms (Δ534–582 or Δ542–582), corresponding to the proteins generated by calpain cleavage at the identified sites (Fig. 6A). The GFP-VGLUT2 fusion protein showed a punctate distribution along neurites, colocalizing with synapsin, a presynaptic marker (Fig. 6B). Many of the GFP-VGLUT2 clusters localized opposite to postsynaptic densities bearing PSD95 (Fig. 6C). These observations indicate that the fusion protein GFP-VGLUT2 presents a synaptic localization similar to endogenous VGLUT2. The colocalization with synapsin (Fig. 6B) and with PSD95 (Fig. 6C) was significantly reduced in the GFP fusion proteins with the truncated form of VGLUT2 (Δ534–582), as assessed by the density, average area and fluorescence intensity of the GFP-VGLUT2 puncta that colocalize with the pre- and post-synaptic markers (Figs. 6B and C). The VGLUT2(Δ542–582) truncated form also showed a reduced colocalization with synapsin and PSD95 when compared with the full-length protein, but this effect was not as significant as that observed for VGLUT2 (Δ534–582). Western blot analysis showed no significant differences in the expression of the GFP fusion proteins with full-length and truncated VGLUT2 (Supplementary Fig. 2A). Analysis of total GFP-VGLUT2 expression showed no significant difference in the number of puncta observed in the neurites of cells transfected with the full-length transporter and the truncated forms. However, there was a reduction in the average area of the puncta containing clustered truncated transporters, and the total fluorescence intensity along neurites was significantly lower when the shorter form of the transporter (Δ534–582) was expressed in hippocampal neurons (Supplementary Fig. 2B).

The *in vitro* cleavage of the fusion protein containing the C-terminus of VGLUT2 suggests that calpain may cleave the VGLUT2 C-terminus at other sites upstream from the identified sites (Fig. 4E). These cleavage events probably generate shorter forms of VGLUT2 which likely will have compromised synaptic localization, as we observed for the GFP-VGLUT2(Δ542–582) and GFP-VGLUT2(Δ534–582) truncated forms. Taken together, the results show that VGLUT2 cleavage significantly reduces its synaptic localization, which may affect glutamatergic synaptic transmission.

Discussion

In the study of cerebral ischemia, not much attention has been paid to the possible changes in vesicular glutamate transporters and their potential roles in this pathology. However, the study on the regulation of these transporters is relevant given their implication in glutamate release. In this work we show that the levels of VGLUT2 are reduced under excitotoxic conditions *in vitro* and *in vivo*, and in transient focal cerebral ischemia, whereas VGLUT1 is downregulated to a smaller extent after *in vitro* toxic stimulation with glutamate, and even up-regulated in excitotoxic conditions *in vivo*. The downregulation of VGLUT2 levels is due to cleavage by calpains, as proven by two types of evidence: i) chemical inhibitors prevented VGLUT2 cleavage in cultured hippocampal neurons subjected to excitotoxic stimulation with glutamate or challenged with OGD; ii) incubation of synaptic vesicles or GST-VGLUT2 C-terminal fusion protein with recombinant calpain induced VGLUT2 cleavage. These findings are in agreement with the key role played by calpains in neuronal death following excitotoxic or ischemic insults (Bevers and Neumar, 2008). Calpains are also activated after MCAO in the cerebral cortex and in the striatum, contributing to neuronal death (Takagaki et al., 1997), and may mediate VGLUT2 cleavage observed in the striatum of mice subjected to this model of ischemia. Likewise, calpains are activated

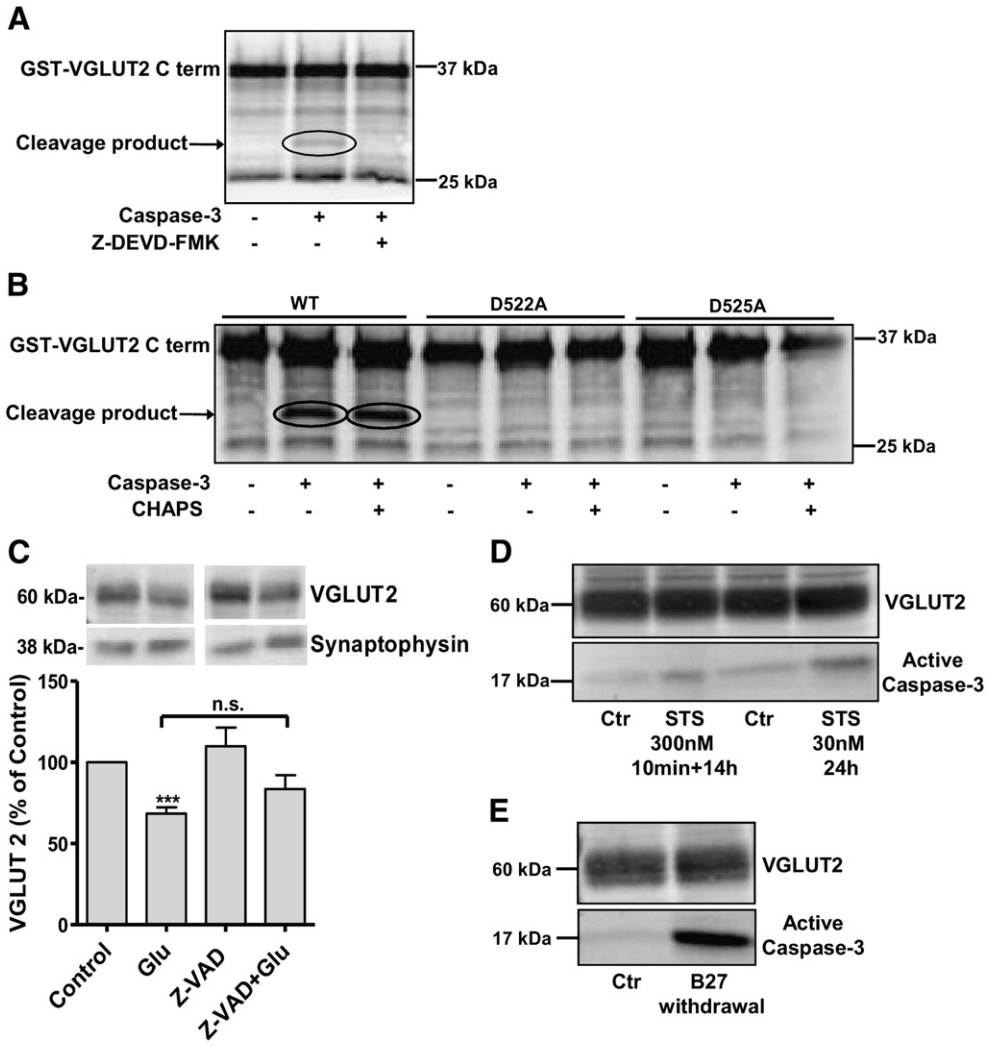

Fig. 5. Characterization of VGLUT2 cleavage by caspase-3. (A) GST-VGLUT2 C-terminus fusion protein was incubated with recombinant caspase-3 (2 U/ml) for 20 h at 37 °C. Z-DEVD-FMK (30 μM) was used to inhibit caspase-3 activity. (B) The consensus site for caspase-3 (DELD) located in VGLUT2 C-terminal (e.g. Fig. 4A) was mutated separately in the two aspartic acid residues to alanine, and the recombinant proteins were incubated with caspase-3 in the presence or absence of CHAPS. Immunoblots were performed using an anti-GST antibody, and the results represent three independent experiments performed in different preparations (A and B). (C) Cultured hippocampal neurons were subjected to excitotoxic stimulation with glutamate (125 μM; 20 min), and VGLUT2 protein levels were determined by western blot 5 h after the insult. In the experiments where the effect of Z-VAD-FMK was tested, the cells were incubated with the inhibitor for 2 h and throughout the experiment. Control (Ctr) VGLUT2 levels were set to 100%. The results are the average ± SEM of 4 independent experiments performed in distinct preparations. Statistical analysis was performed using One Way ANOVA, followed by the Bonferroni's multiple comparison test (n.s.—non significant, $^{***}p<0.001$). (D and E) Cultured hippocampal neurons were treated with staurosporine for 10 min (300 nM) and further incubated in culture conditioned medium for 24 h, or for 24 h (30 nM) (D). In (E) the cells were incubated in culture medium lacking B27 for 48 h. Extracts were immunoblotted using antibodies against VGLUT2 and active caspase-3. The results are representative of three independent experiments performed in different preparations.

after intrahippocampal injection of kainate (Higuchi et al., 2005), and they may be responsible for VGLUT2 cleavage in this model of excitotoxicity *in vivo*. In addition to the involvement of calpains in neurodegeneration, a few reports suggest that calpains also promote survival, by contributing to the activation of the Akt survival pathway (Tan et al., 2006), by inactivating caspases (Chua et al., 2000) or by downregulating NR2 subunits of NMDARs (Kambe et al., 2010).

Our observations showing VGLUT2 downregulation following different excitotoxic or ischemic insults, and upregulation of VGLUT1 after excitotoxicity *in vivo*, are in agreement with previous studies showing that VGLUT2 protein levels are reduced after transient global ischemia in the CA1 layer of the gerbil hippocampus (Kim et al., 2006), and in the cerebral cortex and caudate–putamen of rats subjected to transient MCAO, whereas VGLUT1 levels were increased (Sanchez-Mendoza et al., 2010). Western blot experiments using antibodies against VGLUT2 N- and C-terminal regions showed that the transporter is cleaved at the C-terminal region, and in *vitro* assays led to the identification of two cleavage sites at amino acid residues Asn534 and Lys542.

We also addressed the possibility that caspases might be involved in VGLUT2 downregulation, since these proteases are implicated in ischemic neuronal cell death (Yamashima, 2000) and VGLUT2 contains a consensus DELD sequence that is a target for caspase-3 cleavage. *In vitro* assays showed that VGLUT2 is cleaved by caspase-3 in a manner dependent on the DELD motif present at its C-terminus. However, a pan-caspase inhibitor did not affect significantly the downregulation of VGLUT2 protein levels in hippocampal neurons subjected to excitotoxic conditions. We also used other established cell death stimuli involving a caspase-3 dependent mechanism, such as incubation with staurosporine (Lankiewicz et al., 2000) and trophic factor withdrawal (Guo et al., 2008), which did not promote VGLUT2 downregulation, although caspase-3 activation was observed. Although cleavage of VGLUT2 by caspase-3 may play relevant roles, this was not observed under the experimental conditions tested.

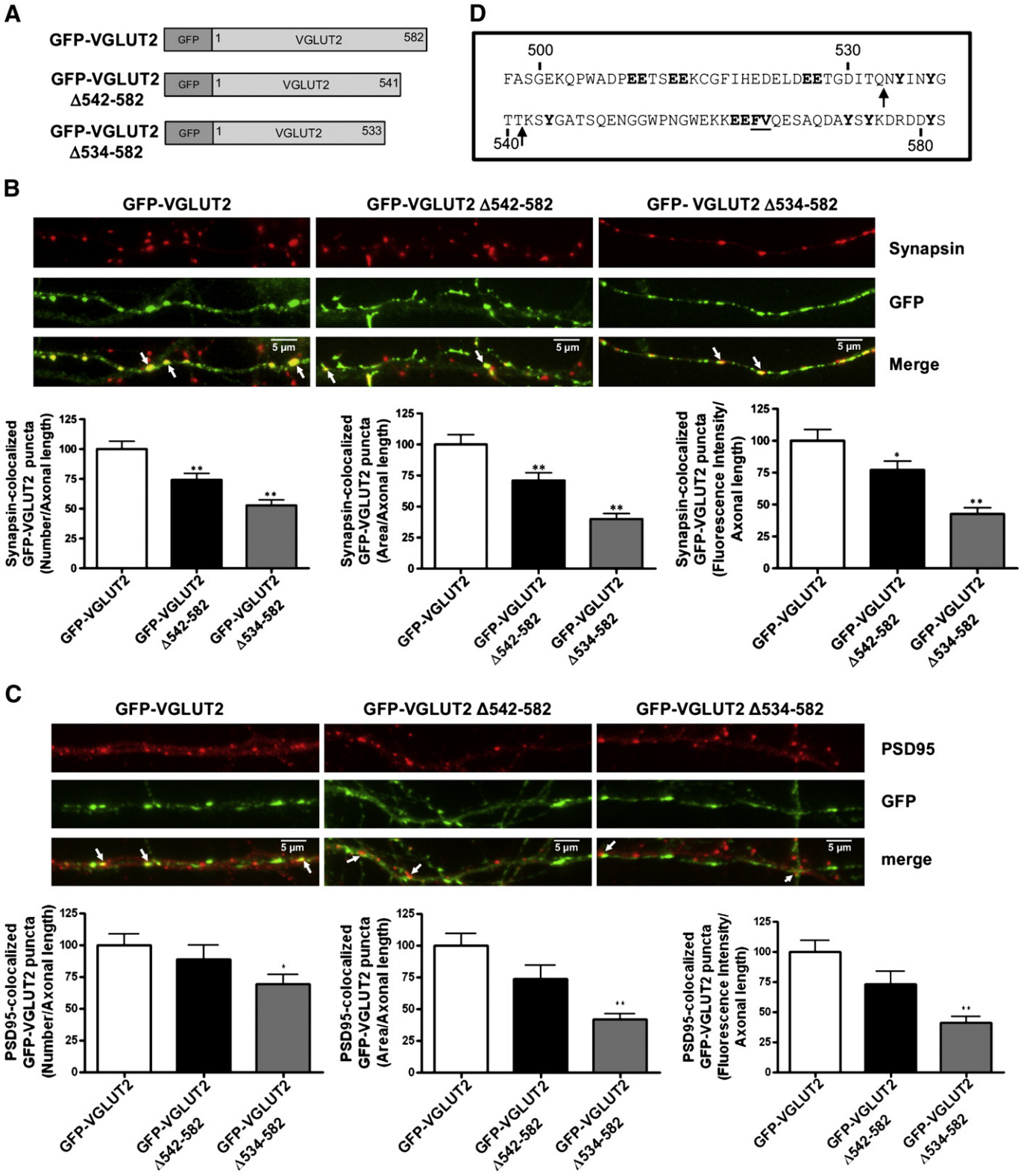

Fig. 6. VGLUT2 cleavage changes the distribution of the protein along the axons. (A) Schematic representation of the GFP-VGLUT2 full-length and truncated constructs. (B and C) Cultured hippocampal neurons were transfected with GFP-VGLUT2, or with the truncated forms Δ534–582 or Δ542–582, and immunocytochemistry was performed using antibodies for GFP (green) and synapsin (B), or GFP and PSD95 (C). Transfected neurons were analyzed for the number, area and fluorescence intensity of GFP-VGLUT2 clusters per axonal length that colocalized with synapsin (B) or with PSD95 (C). The results are presented as % of GFP-VGLUT2 full-length transfected cells, and are the average ± SEM of 3 independent experiments performed in distinct preparations: (B) $n \geq 54$, (C) $n \geq 34$. Statistical analysis was performed using One Way ANOVA, followed by the Dunnett's multiple comparison test ($^{*}p<0.05$, $^{**}p<0.01$). Scale bar: 5 µm. (D) Representation of VGLUT2 C-terminus amino acid sequence, showing in bold putative trafficking signals based on the characterization reported for other vesicular transporters. The arrows indicate the two calpain cleavage sites identified in the present work.

Cleavage of VGLUT2 by calpain gives rise to VGLUT2 forms that are truncated at the C-terminal region. These truncated forms show reduced synaptic localization, which may be related to the removal by calpain cleavage of several amino acid sequences that have been reported to be involved in synaptic delivery and internalization of vesicular transporters, namely a dileucine like (FV) motif, with upstream glutamate clusters, and a tyrosine-based motif (Fig. 6D). The dileucine-like motif is involved in the endocytosis and recycling of VGLUT1 to synaptic vesicles (SVs) in cultured neurons (Voglmaier et al., 2006). In the vesicular monoamine transporter type 2 (VMAT2), a dileucine motif (IL) is required for endocytosis in hippocampal neurons (Li et al., 2005) and sorting to synaptic-like microvesicles (SLMVs) in PC12 cells (Tan et al., 1998). Also, the presence of upstream glutamate residues (EE) is required for VMAT2 sorting into the regulatory secretory pathway (Li et al., 2005). VAchT dileucine motif (LL), and possibly a tyrosine-based motif YNYY, allow internalization and localization to SLMVs (Kim and Hersh, 2004; Tan et al., 1998). Recently, it was reported that deletion of the Drosophila VGLUT C-terminal domain has a consistent effect in reducing internalization and localization to SVs of the transporter (Grygoruk et al., 2010). VGLUT2 also has three putative glycosylation sites at amino acid residues Asn100, Asn101 and Asn470 (UniProtKB Database), which were also correlated with a decrease in VMAT2 location to SVs *in vivo* (Cruz-Muros et al., 2008). Since these sites are conserved in the cleaved VGLUT2, it may explain why the truncated transporter is still found at synapses, although in lower amounts.

Taken together, our results show that ischemic or excitotoxic insults cause VGLUT2 cleavage by calpains, reducing its synaptic targeting, whereas VGLUT1 is not affected by these proteases. Given their implication in glutamate release, a critical step during ischemic brain damage, alterations in VGLUT levels may play an important role in this pathology. The reduction of VGLUT2 protein levels may downregulate glutamate release after an excitotoxic event. Since VGLUT2 has a high expression early in development, when VGLUT1 levels are low (Boulland et al., 2004), the specific decrease of VGLUT2 levels after ischemia might be important in perinatal/neonatal brain injury. Several studies indicate that neonatal mammalian brains are more resistant to ischemic injuries than conspecific adult brains. Strong cellular evidence confirms that neonatal brains possess several strategies that contribute to prevent ischemic injury (Weil et al., 2008). The ischemia-induced reduction of VGLUT2 protein levels may contribute to prevent neonatal ischemic damage.

Supplementary materials related to this article can be found online at doi: 10.1016/j.nbd.2011.07.010.

Acknowledgments

Andrea C. Lobo, João R. Gomes, Tatiana Catarino, Miranda Mele and Ana R. Inácio were supported by FCT, Portugal. This work was supported by FCT and FEDER, Portugal (PTDC/SAU-NEU/65846/2006). We thank Elisabete Carvalho for the assistance in the preparation of cultured hippocampal neurons.

References

Almeida, R.D., Manadas, B.J., Melo, C.V., Gomes, J.R., Mendes, C.S., Graos, M.M., et al., 2005. Neuroprotection by BDNF against glutamate-induced apoptotic cell death is mediated by ERK and PI3–kinase pathways. Cell Death Differ. 12, 1329–1343.
Bevers, M.B., Neumar, R.W., 2008. Mechanistic role of calpains in postischemic neurodegeneration. J. Cereb. Blood Flow Metab. 28, 655–673.
Boulland, J.L., Qureshi, T., Seal, R.P., Rafiki, A., Gundersen, V., Bergersen, L.H., et al., 2004. Expression of the vesicular glutamate transporters during development indicates the widespread corelease of multiple neurotransmitters. J. Comp. Neurol. 480, 264–280.
Broughton, B.R., Reutens, D.C., Sobey, C.G., 2009. Apoptotic mechanisms after cerebral ischemia. Stroke 40, e331–e339.
Candiano, G., Bruschi, M., Musante, L., Santucci, L., Ghiggeri, G.M., Carnemolla, B., et al., 2004. Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis. Electrophoresis 25, 1327–1333.
Chang, H.Y., Yang, X., 2000. Proteases for cell suicide: functions and regulation of caspases. Microbiol. Mol. Biol. Rev. 64, 821–846.
Chao, X.D., Fei, F., Fei, Z., 2010. The role of excitatory amino acid transporters in cerebral ischemia. Neurochem. Res. 35, 1224–1230.
Chua, B.T., Guo, K., Li, P., 2000. Direct cleavage by the calcium-activated protease calpain can lead to inactivation of caspases. J. Biol. Chem. 275, 5131–5135.
Cruz-Muros, I., Afonso-Oramas, D., Abreu, P., Rodriguez, M., Gonzalez, M.C., Gonzalez-Hernandez, T., 2008. Deglycosylation and subcellular redistribution of VMAT2 in the mesostriatal system during normal aging. Neurobiol. Aging 29, 1702–1711.
Doyle, K.P., Simon, R.P., Stenzel-Poore, M.P., 2008. Mechanisms of ischemic brain damage. Neuropharmacology 55, 310–318.
Fremeau Jr., R.T., Voglmaier, S., Seal, R.P., Edwards, R.H., 2004. VGLUTs define subsets of excitatory neurons and suggest novel roles for glutamate. Trends Neurosci. 27, 98–103.
Gascon, S., Sobrado, M., Roda, J.M., Rodriguez-Pena, A., Diaz-Guerra, M., 2008. Excitotoxicity and focal cerebral ischemia induce truncation of the NR2A and NR2B subunits of the NMDA receptor and cleavage of the scaffolding protein PSD-95. Mol. Psychiatry 13, 99–114.
Glazner, G.W., Chan, S.L., Lu, C., Mattson, M.P., 2000. Caspase-mediated degradation of AMPA receptor subunits: a mechanism for preventing excitotoxic necrosis and ensuring apoptosis. J. Neurosci. 20, 3641–3649.
Gomes, J.R., Lobo, A.C., Melo, C.V., Inácio, A.R., Takano, J., Iwata, N., et al., 2011. Cleavage of the vesicular GABA transporter under excitotoxic conditions is followed by accumulation of a truncated VGAT in non-synaptic sites. J. Neurosci. 31, 4622–4635.
Goslin, K., Asmussen, H., Banker, G., 1998. Culturing Nerve Cells. MIT Press, Cambridge.
Grygoruk, A., Fei, H., Daniels, R.W., Miller, B.R., Diantonio, A., Krantz, D.E., 2010. A tyrosine-based motif localizes a *Drosophila* vesicular transporter to synaptic vesicles in vivo. J. Biol. Chem. 285, 6867–6878.
Guo, Z., Jiang, H., Xu, X., Duan, W., Mattson, M.P., 2008. Leptin-mediated cell survival signaling in hippocampal neurons mediated by JAK STAT3 and mitochondrial stabilization. J. Biol. Chem. 283, 1754–1763.
Hertz, L., 2008. Bioenergetics of cerebral ischemia: a cellular perspective. Neuropharmacology 55, 289–309.
Higuchi, M., Tomioka, M., Takano, J., Shirotani, K., Iwata, N., Masumoto, H., et al., 2005. Distinct mechanistic roles of calpain and caspase activation in neurodegeneration as revealed in mice overexpressing their specific inhibitors. J. Biol. Chem. 280, 15229–15237.
Ishida, T., Kinoshita, K., 2008. Prediction of disordered regions in proteins based on the meta approach. Bioinformatics 24, 1344–1348.
Jung, S.K., Morimoto, R., Otsuka, M., Omote, H., 2006. Transmembrane topology of vesicular glutamate transporter 2. Biol. Pharm. Bull. 29, 547–549.
Kambe, Y., Nakamichi, N., Takarada, T., Fukumori, R., Yoneda, Y., 2010. Induced tolerance to glutamate neurotoxicity through down-regulation of NR2 subunits of N-methyl-D-aspartate receptors in cultured rat striatal neurons. J. Neurosci. Res. 88, 2177–2187.
Kim, D.S., Kwak, S.E., Kim, J.E., Jung, J.Y., Won, M.H., Choi, S.Y., et al., 2006. Transient ischaemia affects plasma membrane glutamate transporter, not vesicular glutamate transporter, expressions in the gerbil hippocampus. Anat. Histol. Embryol. 35, 265–270.
Kim, M.H., Hersh, L.B., 2004. The vesicular acetylcholine transporter interacts with clathrin-associated adaptor complexes AP-1 and AP-2. J. Biol. Chem. 279, 12580–12587.
Lankiewicz, S., Marc Luetjens, C., Truc Bui, N., Krohn, A.J., Poppe, M., Cole, G.M., et al., 2000. Activation of calpain I converts excitotoxic neuron death into a caspase-independent cell death. J. Biol. Chem. 275, 17064–17071.
Li, H., Waites, C.L., Staal, R.G., Dobryy, Y., Park, J., Sulzer, D.L., et al., 2005. Sorting of vesicular monoamine transporter 2 to the regulated secretory pathway confers the somatodendritic exocytosis of monoamines. Neuron 48, 619–633.
Nygren, J., Wieloch, T., 2005. Enriched environment enhances recovery of motor function after focal ischemia in mice, and downregulates the transcription factor NGFI-A. J. Cereb. Blood Flow Metab. 25, 1625–1633.
Prehn, J.H., Jordan, J., Ghadge, G.D., Preis, E., Galindo, M.F., Roos, R.P., et al., 1997. Ca2+ and reactive oxygen species in staurosporine-induced neuronal apoptosis. J. Neurochem. 68, 1679–1685.
Rechsteiner, M., Rogers, S.W., 1996. PEST sequences and regulation by proteolysis. Trends Biochem. Sci. 21, 267–271.
Sanchez-Mendoza, E., Burguete, M.C., Castello-Ruiz, M., Gonzalez, M.P., Roncero, C., Salom, J.B., et al., 2010. Transient focal cerebral ischemia significantly alters not only EAATs but also VGLUTs expression in rats: relevance of changes in reactive astroglia. J. Neurochem. 113, 1343–1355.
Stennicke, H.R., Salvesen, G.S., 1997. Biochemical characteristics of caspases-3, -6, -7, and -8. J. Biol. Chem. 272, 25719–25723.
Szydlowska, K., Tymianski, M., 2010. Calcium, ischemia and excitotoxicity. Cell Calcium 47, 122–129.
Takagaki, Y., Itoh, Y., Aoki, Y., Ukai, Y., Yoshikuni, Y., Kimura, K., 1997. Inhibition of ischemia-induced fodrin breakdown by a novel phenylpyrimidine derivative NS-7: an implication for its neuroprotective action in rats with middle cerebral artery occlusion. J. Neurochem. 68, 2507–2513.
Takano, J., Tomioka, M., Tsubuki, S., Higuchi, M., Iwata, N., Itohara, S., et al., 2005. Calpain mediates excitotoxic DNA fragmentation via mitochondrial pathways in adult brains: evidence from calpastatin mutant mice. J. Biol. Chem. 280, 16175–16184.
Tan, P.K., Waites, C., Liu, Y., Krantz, D.E., Edwards, R.H., 1998. A leucine-based motif mediates the endocytosis of vesicular monoamine and acetylcholine transporters. J. Biol. Chem. 273, 17351–17360.

Tan, Y., Wu, C., De Veyra, T., Greer, P.A., 2006. Ubiquitous calpains promote both apoptosis and survival signals in response to different cell death stimuli. J. Biol. Chem. 281, 17689–17698.
Tompa, P., Buzder-Lantos, P., Tantos, A., Farkas, A., Szilagyi, A., Banoczi, Z., et al., 2004. On the sequential determinants of calpain cleavage. J. Biol. Chem. 279, 20775–20785.
Voglmaier, S.M., Kam, K., Yang, H., Fortin, D.L., Hua, Z., Nicoll, R.A., et al., 2006. Distinct endocytic pathways control the rate and extent of synaptic vesicle protein recycling. Neuron 51, 71–84.
Weil, Z.M., Norman, G.J., DeVries, A.C., Nelson, R.J., 2008. The injured nervous system: a Darwinian perspective. Prog. Neurobiol. 86, 48–59.
Wojcik, S.M., Rhee, J.S., Herzog, E., Sigler, A., Jahn, R., Takamori, S., et al., 2004. An essential role for vesicular glutamate transporter 1 (VGLUT1) in postnatal development and control of quantal size. Proc. Natl Acad. Sci. USA 101, 7158–7163.
Xu, W., Wong, T.P., Chery, N., Gaertner, T., Wang, Y.T., Baudry, M., 2007. Calpain-mediated mGluR1alpha truncation: a key step in excitotoxicity. Neuron 53, 399–412.
Yamashima, T., 2000. Implication of cysteine proteases calpain, cathepsin and caspase in ischemic neuronal death of primates. Prog. Neurobiol. 62, 273–295.
Yuen, E.Y., Liu, W., Yan, Z., 2007. The phosphorylation state of GluR1 subunits determines the susceptibility of AMPA receptors to calpain cleavage. J. Biol. Chem. 282, 16434–16440.